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APPLICATION

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TITLE: RECOMBINANT HUMAN ALPHA-FETOPROTEIN AS A CELL PROLIFERATIVE AGENT

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RECOMBINANT HUMAN ALPHA-FETOPROTEIN AS A CELL PROLIFERATIVE AGENT

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Background of the Invention

This invention relates to cell growth and cell culture.

Mammalian alpha-fetoprotein (AFP) is a 70,000-Da glycoprotein of primarily yolk sac and hepatic origin which is present in fetal blood in milligram amounts during perinatal life. At birth serum AFP levels begin a gradual decline to the low nanogram amounts normally found in the adult. Chemical analysis has shown that AFP molecules are composed of single polypeptide chains containing about 4% carbohydrate.

Summary of the Invention

I have discovered that unglycosylated recombinant human alpha-fetoprotein made in a prokaryote (e.g., E. coli) is a cell proliferative agent, e.g., promotes the growth of bone marrow *in vitro*.

In general, the invention features a cell culture medium including recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof. Preferably, such recombinant human alpha-fetoprotein is produced in a prokaryotic cell (E. coli) and is unglycosylated.

Accordingly, the invention features a method of cell culture, said method including (a) providing a cell culture medium including recombinant human alpha-fetoprotein; (b) providing a cell; (c) and growing the cell in the medium, where the cell proliferates, and is maintained. Preferably, the cell is a mammalian cell. Examples of such mammalian cells include bone marrow cells (e.g., a T cells, a natural

killer cell, a lymphocyte, etc.), hybridomas or a genetically-engineered cell line. Examples of other cells include hematopoietic cells such as stem cells, blast cells, progentior cells (e.g., erythroid progenitor cells such as burst-forming units and colony-forming units), myeloblasts, macrophages, monocytes, macrophages, lymphocytes, T-lymphocytes, B-lymphocytes, eosinophils, basophils, tissue mast cells, megarkaryocytes (see e.g., *Best and Taylor's Physiological Basis of Medical Practice*, John B. West, ed., Williams & Wilkins, Baltimore). In other preferred embodiments the method involves ex vivo cell culture.

In another aspect, the invention features a method for inhibiting myelotoxcity in a mammal (e.g., a human patient) involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or a myelotoxic-inhibiting analog or fragment thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (E. coli) and is unglycosylated.

In another aspect, the invention features a method of inhibiting suppression of bone marrow cell proliferation in a mammal, the method involving administering to the mammal an effective amount of recombinant alpha-fetoprotein or an anti-suppressive fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of promoting bone marrow cell proliferation in a mammal, involving administering to the mammal an effective amount of recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof. Preferably, the recombinant

human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of preventing bone marrow cell transplantation rejection in a mammal, involving administering to the mammal an effective amount of recombinant human alpha-fetoprotein or an anti-rejection fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

By "cell-stimulating" is meant increasing cell proliferation, increasing cell division, promoting cell differentiation and/or development, or promoting cell longevity.

By "therapeutically effective amount" is meant a dose of unglycosylated recombinant human alpha-fetoprotein or an cell-stimulating fragment or analog thereof capable of stimulating the proliferation of a cell.

By "recombinant human alpha-fetoprotein" is meant a polypeptide having substantially the same amino acid sequence as the protein encoded by the human alpha-fetoprotein gene (Fig. 1, SEQ ID NO: 1) as described by Morinaga et al., Proc. Natl. Acad. Sci., USA 80: 4604 (1983). The method of producing recombinant human alpha-fetoprotein in a prokaryotic cell is described in ~~U.S. Ser. No. 08/133,773 issuing as~~ U.S. Pat. No. 5,384,250.

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By "myelotoxic-inhibiting" is meant inhibiting myeloablation.

According to the invention, administration of recombinant human alpha-fetoprotein ("rHuAFP") (or a fragment or analog thereof) can be an effective means for promoting and boosting cell growth in vitro, ex vivo, or in vivo. Administration of such rHuAFP can also be an

effective means of preventing or treating or ameliorating myleotoxcemia in a mammal.

The use of rHuAFP is especially advantageous since there are no known adverse side effects related to human alpha-fetoprotein and it is believed that relatively high doses can be safely administered. Furthermore, the use of rHuAFP as a principal component of tissue culture media is advantageous since there is little potential for contamination with pathogens.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The drawing will first be described.

Drawings

Fig. 1 is the nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of the cDNA encoding human alpha-fetoprotein.

Fig. 2 is the SDS-PAGE analysis of rHuAFP Fragment I (SEQ ID NO:3) (Lane A, MW marker; Lane B, natural human alpha-fetoprotein (AFP); Lane C, unpurified rHuAFP; Lane D, rHuAFP Fragment I, and Lane E, rHuAFP (amino acids 1- 590 of Fig. 1, SEQ ID NO:2).

Fig. 3 is a bar graph showing murine bone marrow proliferation in serum-free RPMI medium in the presence or absence of both 400 μg/ml rHuAFP and 5μg/ml transferrin.

Production of Recombinant Human Alpha-Fetoprotein

As summarized above, the invention includes therapies for promoting cell proliferation and for the prevention and treatment of a myelotoxic condition involving administering recombinant human alpha-fetoprotein ("rHuAFP")

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or fragments or analogs thereof. Methods for producing such rHuAFP in a prokaryotic cell are described in ~~U.S. Ser. 08/133,773 issuing as~~ U.S. Patent No. 5,384,250. Methods for producing rHuAFP fragments and analogs will now be discussed in greater detail.

Fragments and Analogs

The invention includes biologically active fragments of rHuAFP. A biologically active fragment of rHuAFP is one that possesses at least one of the following activities: (a) directs a specific interaction with a target cell, e.g., binds to a cell expressing a receptor which is recognized by rHuAFP (e.g., the membrane of a bone marrow cell); or (b) stimulates, increases, expands or otherwise causes the proliferation of a cell (e.g., binds to a cell surface receptor to impart an proliferative or stimulating-signal). The ability of rHuAFP fragments or analogs to bind to a receptor which is recognized by rHuAFP can be tested using any standard binding assay known in the art. Biological activity of a rHuAFP fragment or analog can also be tested according to standard methods, e.g., those described herein.

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In general, fragments of rHuAFP are produced according to the techniques of polypeptide expression and purification described in ~~U.S. Ser. No. 08/133,773 (~~U.S. Patent No. 5,384,250~~)~~. For example, suitable fragments of rHuAFP can be produced by transformation of a suitable host bacterial cell with part of an HuAFP-encoding cDNA fragment (e.g., the cDNA described above) in a suitable expression vehicle. Alternatively, such fragments can be generated by standard techniques of PCR and cloned into the expression vectors (<u>supra</u>). Accordingly, once a fragment of rHuAFP is expressed, it may be isolated by various chromatographic and/or immunological methods known in the art. Lysis and fractionation of rHuAFP-containing cells prior to affinity

chromatography may be performed by standard methods. Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, Work and Burdon, eds., Elsevier, 1980).

A rHuAFP fragment may also be expressed as a fusion protein with maltose binding protein produced in E. coli. Using the maltose binding protein fusion and purification system (New England Biolabs), the cloned human cDNA sequence can be inserted downstream and in frame of the gene encoding maltose binding protein (malE), and the malE fusion protein can then be overexpressed. In the absence of convenient restriction sites in the human cDNA sequence, PCR can be used to introduce restriction sites compatible with the vector at the 5′ and 3′ end of the cDNA fragment to facilitate insertion of the cDNA fragment into the vector.

Following expression of the fusion protein, it can be purified by affinity chromatography. For example, the fusion protein can be purified by virtue of the ability of the maltose binding protein portion of the fusion protein to bind to amylose immobilized on a column.

To facilitate protein purification, the pMalE plasmid contains a factor Xa cleavage site upstream of the site into which the cDNA is inserted into the vector. Thus, the fusion protein purified as described above can then be cleaved with factor Xa to separate the maltose binding protein from a fragment of the recombinant human cDNA gene product. The cleavage products can be subjected to further chromatography to purify rHuAFP from the maltose binding protein. Alternatively, a fragment of rHuAFP may be expressed as a fusion protein containing a polyhistidine tag can be produced. Such an alpha-fetoprotein fusion protein

may then be isolated by binding of the polyhistidine tag to an affinity column having a nickel moiety which binds the polyhistidine region with high affinity. The fusion protein may then be eluted by shifting the pH within the affinity column. The rHuAFP can be released from the polyhistidine sequences present in the resultant fusion protein by cleavage of the fusion protein with specific proteases.

Recombinant HuAFP fragment expression products (e.g., produced by any of the prokaryotic systems described in U.S. 5,384,250 ~~No. Ser. 08/133,773~~) may be assayed by immunological procedures, such as Western blot, immunoprecipitation analysis of recombinant cell extracts, or immunofluorescence (using, e.g., the methods described in Ausubel et al., *Current Protocols In Molecular Biology*, Greene Publishing Associates and Wiley Interscience (John Wiley & Sons), New York, 1994).

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Once a fragment of rHuAFP is expressed, it is isolated using the methods described supra. Once isolated, the fragment of rHuAFP can, if desired, be further purified by using the techniques described supra. Fragments can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, Il).

The ability of a candidate rHuAFP fragment to exhibit a biological activity of alpha-fetoprotein is assessed by methods known to those skilled in the art (e.g., those described herein).

The purified recombinant gene product or fragment thereof can then be used to raise polyclonal or monoclonal antibodies against the human recombinant alpha-fetoprotein using well-known methods (see Coligan et al., eds., *Current Protocols in Immunology*, 1992, Greene Publishing Associates and Wiley-Interscience). To generate monoclonal antibodies,

a mouse can be immunized with the recombinant protein, and antibody-secreting B cells isolated and immortalized with a non-secretory myeloma cell fusion partner. Hybridomas are then screened for production of recombinant human alpha-fetoprotein (or a fragment or analog thereof)-specific antibodies and cloned to obtain a homogenous cell population which produces monoclonal antibodies.

As used herein, the term "fragment," as applied to a rHuAFP polypeptide, is preferably at least 20 contiguous amino acids, preferably at least 50 contiguous amino acids, more preferably at least 100 contiguous amino acids, and most preferably at least 200 to 400 or more contiguous amino acids in length. Fragments of rHuAFP molecules can be generated by methods known to those skilled in the art, e.g., proteolytic cleavage or expression of recombinant peptides, or may result from normal protein processing (e.g., removal of amino acids from nascent polypeptide that are not required for biological activity).

Recombinant HuAFP fragments of interest include, but are not limited to, Domain I (amino acids 1 (Thr) - 197 (Ser), see Fig. 1, SEQ ID NO:3), Domain II (amino acids 198 (Ser) - 389 (Ser), see Fig. 1, SEQ ID NO:4), Domain III (amino acids 390 (Gln) - 590 (Val), see Fig. 1, SEQ ID NO: 5), Domain I+II (amino acids 1 (Thr) - 389 (Ser), see Fig. 1, SEQ ID NO:6), Domain II+III (amino acids 198 (Ser) - 590 (Val), see Fig. 1, SEQ ID NO: 7), and rHuAFP Fragment I (amino acids 266 (Met) - 590 (Val), see Fig. 1, SEQ ID NO:8). Activity of a fragment is evaluated experimentally using conventional techniques and assays, e.g., the assays described herein.

The invention further includes analogs of full-length rHuAFP or fragments thereof. Analogs can differ from rHuAFP by amino acid sequence differences, or by

modifications (e.g., post-translational modifications) which do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 80%, more preferably 85%, and most preferably 90% or even 99% amino acid identity with all or part of a rHuAFP amino acid sequence. Modifications (which do not normally alter primary sequence) include <u>in vivo</u>, or <u>in vitro</u> chemical derivatization of polypeptides, e.g., acetylation, or carboxylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally occurring rHuAFP by alterations in primary sequence, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1989, or Ausubel et al., <u>supra</u>). Also included are cyclized peptide molecules and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids, or L-amino acids with non-natural side chains (see e.g., Noren et al., <u>Science</u> 244:182, 1989). Methods for site-specific incorporation of non-natural amino acids into the protein backbone of proteins is described, e.g., in Ellman et al., <u>Science</u> 255:197, 1992. Also included are chemically synthesized polypeptides or peptides with modified peptide bonds (e.g., non-peptide bonds as

described in U.S. Pat. No. 4,897,445 and U.S. Pat. No. 5,059,653) or modified side chains to obtain the desired pharmaceutical properties as described herein. Useful mutants and analogs are identified using conventional methods, e.g., those described herein.

The cloning, expression, isolation and characterization of exemplary rHuAFP fragments now follows. These examples are provided to illustrate, not limit, the invention.

EXPERIMENTAL

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) rHuAFP Fragments

Plasmid constructs encoding fragments of human alpha-fetoprotein were prepared using polymerase chain reaction (PCR) techniques known to those skilled in the art of molecular biology, using oligonucleotide primers designed to amplify specific portions of the human alpha-fetoprotein gene (see e.g., *PCR Technology*, H.A. Erlich, ed., Stockton Press, New York, 1989; *PCR Protocols: A Guide to Methods and Applications*, M.A. Innis, David H. Gelfand, John J. Sninsky, and Thomas J. White, eds., Academic Press, Inc., New York, 1990, and Ausubel et. al., supra).

The following six rHuAFP fragments were prepared to evaluate their biological activity (e.g., according to the methods disclosed herein):

a Domain I	Amino acids 1 (Thr) - 197 (Ser),	(Fig. 1, SEQ ID NO: 3)
a Domain II	Amino acids 198 (Ser) - 389 (Ser),	(Fig. 1, SEQ ID NO: 4)
a Domain III	Amino acids 390 (Gln) - 590 (Val),	(Fig. 1, SEQ ID NO: 5)
a Domain I + II	Amino acids 1 (Thr) - 389 (Ser),	(Fig. 1, SEQ ID NO: 6)
a Domain II + III	Amino acids 198 (Ser) - 590 (Val),	(Fig. 1, SEQ ID NO: 7)
a rHuAFP Fragment I	Amino acids 266 (Met) - 590 (Val),	(Fig. 1, SEQ ID NO: 8)

Amino acid sequences were deduced from those shown for human alpha-fetoprotein (1 (Thr) - 590 (Val), SEQ ID NO:2) in Fig. 1. Fragments of rHuAFP designated Domain I, Domain II, Domain III, Domain I+II, Domain II+III and rHuAFP Fragment I were synthesized using standard PCR reaction conditions in 100 μL reactions containing 34 μL H_2O, 10 μL 10X reaction buffer, 20 μL 1 mM dNTP, 2 μL DNA template (HuAFP cloned in pI18), appropriate 5′ and 3′ oligonucleotide primers (10 μL 10 pmol/μL 5′ primer, 10 μL 10 pmol/μL 3′ primer), 1 μL glycerol, 10 μL DMSO, and 1 μL *Pfu* polymerase (Stratagene, LaJolla, CA). Primers used for PCR amplifications were:

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DomI25	5′-AAAAAAGGTACCACACTGCATAGAAATGAA-3′	(SEQ ID NO:9)
DomI3	5′-AAAAAAGGATCCTTAGCTTTCTCTTAATTCTTT-3′	(SEQ ID NO:10)
DomII5	5-′AAAAAAATCGATATGAGCTTGTTAAATCAACAT-3′	(SEQ ID NO: 11)
DomII3	5′-AAAAAAGGATCCTTAGCTCTCCTGGATGTATTT-3′	(SEQ ID NO:12)
DomIII5	5′-AAAAAAATCGATATGCAAGCATTGGCAAAGCGA-3′	(SEQ ID NO:13)
DomIII3	5′-AAAAAAGGATCCTTAAACTCCCAAAGCAGCACG-3′	(SEQ ID NO:14)
5′rHuAFP Fragment I	5′-AAAAAAATCGATATGTCCTACATATGTTCTCAA-3′	(SEQ ID NO:15)

Accordingly, primer pairs DomI25 and DomI3, DomII5 and DomII3, DomIII5 and DomIII3, 5′rHuAFP Fragment I and DomIII3, DomI25 and DomII3, and DomII5 and DomIII3 were used to isolate cDNA sequences of Domain I, Domain II, Domain III, rHuAFP Fragment I, Domain I+II, and Domain II+III, respectively, of rHuAFP. Annealing, extension, and denaturation temperatures were 50°C, 72°C, and 94°C, respectively, for 30 cycles. PCR products were purified according to standard methods. Purified PCR products encoding Domain I and Domain I+II were digested individually with KpnI and BamHI and cloned separately into KpnI/BamHI-treated pTrp4. Purified PCR products encoding Domain II, Domain III, Domain II+III, and rHuAFP Fragment I were digested individually with Bsp106I and BamHI and were cloned

separately into Bsp106I/BamHI-treated pTrp4. Each plasmid construct was subsequently transformed into competent E. coli cells. Since the expression product will begin with the amino acid sequence encoded by the translation start signal methionine, it is expected that such signal will be removed, or in any event, not affect the bioactivity of the ultimate expression product.

RESULTS

Expression and Purification

E. coli containing the expression plasmid encoding rHuAFP Fragment I was cultured and purified. Fig. 2 (lane D) shows the SDS-PAGE profile of the purified rHuAFP Fragment I. N-terminal amino acid sequence analysis showed that rHuAFP Fragment I possessed the amino acid sequence Ser_{267}-Tyr-Ile-Cys-Ser-Gln-Gln-Asp-Thr_{275} (SEQ ID NO:16) which corresponds to the expected N-terminal amino acid sequence of rHuAFP Fragment I (see Fig. 1, SEQ ID NO:8) where the initiating methionine is cleaved intracellularly.

Cell Culture Media

The invention provides a media containing rHuAFP (or a fragment or analog thereof) for cell culture. While media of the invention generally does not require the use of serum (e.g., fetal bovine serum, calf serum, horse serum, normal mouse serum, human serum, porcine serum, rabbit serum etc.), since such rHuAFP is intended to replace or supplement the use of serum, those skilled in the art will understand and recognize that serum can be added if desired. Media formulations are generally prepared according to methods known in the art. Accordingly, any standard medium, e.g., RMPI-1630 Medium, CMRL Medium, Dulbecco's Modified Eagle Medium (D-MEM), Fischer's Medium, Iscove's Modified

Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, NCTC Medium, and the like can be formulated with rHuAFP (or a fragment or analog thereof) at the desired effective concentration. If desired, media supplements, e.g., salt solutions (e.g., Hank's Balanced Salt Solution or Earle's Balanced Salt Solution), antibiotics, nucleic acids, amino acids, carbohydrates, and vitamins are added according to known methods. If desired, growth factors, colony-stimulating factors, cytokines and the like can also be added to media according to standard methods. For example, media of the invention can contain any of the following substances, alone or in combination, with rHuAFP (or a fragment or analog thereof): erythropoietin, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), an interleukin (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, etc.), insulin-growth factor (IGF), transferrin, albumin, and stem-cell growth factor (SCF). Media of the invention are useful for culturing a variety of eukaryotic cells, e.g., mammalian cells, yeast cells, amphibian cells, and insect cells. Media can also be used for culturing any tissue or organ. Such media can also be used in a variety of culture conditions and for a variety of biological applications. Examples of such culture conditions include, without limitation, bioreactors (e.g., continuous or hollow fiber bioreactors), cell-suspension cultures, semisolid cultures, liquid cultures, and long-term cell suspension cultures. Media of the invention are also useful for industrial applications, e.g., culturing hybridoma cells, genetically-engineered mammalian cells, tissues or organs.

Recombinant Human Alpha-Fetoprotein As A Cell-Proliferative Agent

Cell growth-promoting attributes of rHuAFP (or a fragment or analog thereof) is evaluated by any standard assay for analysis of cell proliferation *in vitro* and *in vivo*. As discussed *infra*, the art provides animal systems for *in vivo* testing of cell growth promoting or boosting characteristics of rHuAFP (or a fragment or analog thereof). Furthermore, a wide variety of *in vitro* systems are also available for testing growth-promoting or growth-boosting aspects of rHuAFP (or a fragment or analog thereof).

Any cell that proliferates in response to rHuAFP (or a fragment or analog thereof) can be identified according to standard methods known in the art. For example, proliferation of a cell (e.g., a bone marrow cell) can be monitored by culturing in a liquid media containing the test compound, either alone or in combination with other growth factors, added artificially to a serum-free or serum-based medium. Alternatively, such bone marrow cells can be cultured in a semisolid matrix of dilute agar or methylcellulose, and the test compound, alone or in combination with other growth factors, can be added artificially to a serum-free or serum-reduced medium. In the semisolid matrix the progeny of an isolated precursor cell, proliferating in response to rHuAFP or a fragment or analog thereof, remain together as a distinguishable colony. For example, a bone marrow cell may be seen to give rise to a clone of a plurality of bone marrow cells, e.g., NK cells. Such culture systems provide a facile way for assaying whether a cell responds to rHuAFP (a fragment or analog thereof) either alone or in combination with other growth factors.

If desired, identification and separation of expanded subpopulations of cells is performed according to standard methods. For example, cells may be analyzed by fluorescence-activated cell sorting (FACS). This procedure generally involves labelling cells with antibodies coupled to a fluorescent dye and separating the labeled cells from the unlabelled cells in a FACS, e.g., FACScan (Becton Dickson). Thus virtually any cell can be identified and separated, e.g., by analyzing the presence of cell surface antigens (see e.g., Shah et al., J. Immunol. 140:1861, 1988). When a population of cells is obtained, it is then analyzed biochemically or, alternatively, provides a starting population for additional cell culture, allowing the action of the cells to be evaluated under defined conditions in culture.

In one working example, the effect of rHuAFP (or a fragment or analog thereof) on the growth of human bone marrow cells is examined as follows. In general, human bone marrow samples are obtained according to standard procedures after informed consent. For example, bone marrow is obtained from the iliac crest of a healthy donor and the marrow cells are diluted in phosphate-buffered saline at room temperature. Cells are then washed and cultured in an appropriate growth medium. For example, cultures can be set up by inoculating bone marrow cells in 20-30 ml of McCoy's medium containing 50 U/ml penicillin, 50 U/ml streptomycin and 2 mM L-glutamine. Cultures are incubated in the presence or absence of the test compound alone, or in combination with other growth factors, e.g., transferrin or GM-CSF. The cultures are subsequently incubated at 37°C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2 for the desired time period. Cell proliferation assays are performed according to standard methods. For example,

replicate samples cultured in the presence and absence of the test compound are analyzed by pulsing the cells with 1-2 μCi of ^{3}HTdR. After an incubation period, cultures are harvested onto glass-fiber filters and the incorporated ^{3}H measured by liquid scintillation. Comparative studies between treated and control cells, e.g., cell cultured in the presence of rHuAFP versus cells cultured in the absence of rHuAFP, are used to determine the relative efficacy of the test molecule in stimulating cell proliferation. A molecule which stimulates cell proliferation is considered useful in the invention.

To evaluate the proliferative effects of rHuAFP (or a fragment or analog thereof) e.g., the effect of the test compound on hematopoiesis _in vivo_, the test molecule is administered to sublethally irradiated mice (or mice treated with an immunosuppressive agent such as cyclosporine or FK-506, or a chemotherapeutic agent such as 5-fluorouracil or cyclophosphamide or any other method known in the art to deplete bone marrow) and normal mice according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration of the test compound to treated mice is initiated prior to and/or after treating the animal, e.g., with sublethal radiation or immunotherapy or chemotherapy. Control animals receive a placebo, e.g., human serum albumin or diluent, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on hematopoiesis is monitored by standard techniques. For example, white blood cell count in peripheral blood and spleen in both treated and control animals are analyzed. Qualitative and quantitative analyses of bone marrow, e.g., lymphocytic lineage or myeloid lineage or any other cell type, can also be determined and analyzed according to conventional

methods. Comparative data between treated and control animals are used to determine the relative efficacy of the test molecule in promoting cell proliferation, e.g., stimulates bone marrow cell production, mature B lymphocyte, thymocyte, or peripheral T lymphocyte cell production. A test molecule which stimulates cell proliferation is considered useful in the invention.

The following example demonstrates that unglycosylated rHuAFP stimulates the growth of bone marrow cells _in vitro_. This example is provided to illustrate, not limit, the invention.

EXPERIMENTAL

MATERIALS AND METHODS

Animals

Adult male and female CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All mice were bred and maintained in our animal facility. Animals used in this study were 12 to 20 weeks old.

Cultures

Bone marrow cells were collected by flushing the tibias and femurs of CBA/J mice with modified Dulbecco's phosphate-buffered saline (PBS) using a sterile syringe and 25-gauge needle. Homogenous single-cell suspensions were obtained by the repeated passage of cell mixtures through a Pasteur pipet. All cells were washed twice by centrifugation at 250g for 10 min in PBS and then assessed for viability by trypan blue dye exclusion. A cell viability of 95% or better was recorded in all experiments. Cells were then adjusted to the desired concentration prior to use. Bone marrow cells (250,000) were cultured in 96-well round-bottom microtiter plates (Flow Laboratories, Mississauga, Ontario, Canada). The culture medium was

serum-free RPMI plus 4 mM L-glutamine, 20 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO Laboratories, Burlington, Ontario, Canada), 5 μg/ml transferrin, and 5 X 10^{-5} 2-mercaptoethanol (Eastman Chemicals Co., Rochester N.Y.). Cells were cultured in the presence or absence of rHuAFP at a concentration of 400 μg/ml, respectively. Total volume of all cultures was 0.2 ml. Cultures were maintained at 37°C in 95% humidified air and 5% CO_2. Six hours prior to harvesting, the cultures were pulsed with 1 μCi tritiated thymidine (NEN, sp act 77.1 Ci/mmol). Cells were then harvested on glass fiber mats (Flow Labs) with a multiple sample harvester (Skatron, Flow Labs). Water-insoluble tritiated thymidine incorporation was measured with an LKB 1215 Rackbeta II using standard liquid scintillation techniques.

<u>rHuAFP</u>

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Recombinant HuAFP was synthesized and purified using the methods described in ~~U.S. Ser. No. 08/133,773 (~~U.S. Pat. No. 5,384,250~~)~~. Alternatively, rHuAFP can be obtained from Immtek, Inc. (Boston, MA).

RESULTS

Effects of rHuAFP on Bone Marrow Proliferation in Serum-Free Media

The effects of purified rHuAFP on cultured murine bone marrow was evaluated in serum-free medium. In this experiment, 2.5 X 10^5 viable cells from bone marrow of CBA/J mice were cultured for 72 hours in serum-free RPMI media in the presence or absence of rHuAFP at a final concentration of 400 μg/ml and transferrin at a final concentration of 5 μg/ml. Data shown in Fig. 3 indicate that bone marrow cells undergo a strong proliferative response in the presence of unglycosylated rHuAFP; with a stimulation index (SI) of 35.

No such proliferation was observed when bone marrow cells were cultured in the absence of rHuAFP.

Therapy

As demonstrated above, rHuAFP is effective in promoting the proliferation of cells and accordingly is useful for therapy involving the promotion of cell proliferation, e.g., proliferation of bone marrow cells, and in treatment for the prevention of side effects of immunosuppressive therapy, radiotherapy or chemotherapy, or other therapies known to depress the immune system and suppress bone marrow production, causing myelotoxicity. Accordingly, rHuAFP (or a fragment or analog thereof) is employed to treat deficiencies in hematopoietic progenitor or stem cells, or related disorders. Recombinant HuAFP (or a fragment or analog thereof) may also be employed in methods for treating cancer and other pathological states resulting in myelotoxcity, exposure to radiation or drugs, and including for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies, including immune cell or hematopoietic cell deficiency following autologous or non-autologous bone marrow transplantation. Recombinant HuAFP (or a fragment or analog thereof) may also be employed to stimulate development of megakaryocytes and natural killer cells in vitro or in vivo.

The media, compositions, and methods of the invention are also useful for treating cancers that are treated by bone marrow transplants (BMT) that involve removing bone marrow cells from the patient, maintaining these cells in an ex vivo culture while the patient is treated with radiation or chemotherapy, and then transplanting these cells back into the patient after the treatment has been completed to restore the patient's bone

marrow. Accordingly, rHuAFP may be employed for BMT as a means for reconstituting bone marrow in *ex vivo* cell culture medium and for promoting bone marrow cell proliferation *in vivo*. Recombinant HuAFP (a fragment or analog thereof) is also useful for other cell therapies, e.g. cell expansion and/or gene therapy protocols, therapies requiring *ex vivo* cell culture. Recombinant HuAFP (a fragment or analog) is also useful in the prevention of autologous or allogenic bone marrow transplant rejection.

Therapeutic Administration

Recombinant HuAFP (or a fragment or analog thereof) can be formulated according to known methods to prepare pharmaceutically useful compositions. Recombinant human alpha-fetoprotein, e.g., rHuAFP (or a fragment or analog thereof), is preferably administered to the patient in an amount which is effective in preventing or ameliorating the symptoms of myleotoxcity. Generally, a dosage of 0.1 ng/kg to 10 g/kg body weight is adequate. For example, treatment of human patients will be carried out using a therapeutically effective amount of rHuAFP (or a fragment or analog thereof) in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's *Pharmaceutical Sciences* by E.W. Martin. The amount of rHuAFP to be administered will vary depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, and size of the patient predisposed to or suffering from the disease. Preferable routes of administration include, for example, oral, subcutaneous, intravenous, intrperitoneally, intramuscular, transdermal or intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, rHuAFP can be given to a patient by injection or

implantation of a slow release preparation, for example, in a slowly dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example, those described above). Alternatively, rHuAFP can be administered using an external or implantable infusion pump, thus allowing a precise degree of control over the rate of drug release, or through installation of rHuAFP in the nasal passages in a similar fashion to that used to promote absorption of insulin. As an alternative to nasal transmucosal absorption, rHuAFP can be delivered by aerosol deposition of the powder or solution into the lungs.

The therapeutic method(s) and compositions of the present invention may also include co-administration with other human growth factors. Exemplary cytokines or hematopoietins for such use include, without limitation, factors such as an interleukin (e.g., IL-1), GM-CSF, G-CSF, M-CSF, tumor necrosis factor (TNF), transferrin, and erythropoietin. Growth factors like B cell growth factor, B cell differentiation factor, or eosinophil differentiation factors may also prove useful in co-administration with rHuAFP (or a fragment or analog thereof). The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

Treatment is started generally with the diagnosis or suspicion of myelotoxcity and is generally repeated on a regular or daily basis to ameliorate or prevent the progression or exacerbation of the condition. Protection or prevention from the development of a myleotoxcemic condition is also achieved by administration of rHuAFP prior to the onset of the disease. If desired, the efficacy of the

treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for myelotoxcity.

The method(s) of the invention can also be used to treat non-human mammals, for example, domestic pets, or livestock.

All publications, manufacturer's instructions, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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What is claimed is: